

Late Stage Polyribitol Phosphate Wall Teichoic Acid Biosynthesis in *Staphylococcus aureus*

Timothy C. Meredith<sup>†</sup>, Jonathan G. Swoboda<sup>†</sup>, and Suzanne Walker<sup>†\*</sup>

<sup>†</sup>Department of Microbiology and Molecular Genetics  
Harvard Medical School, Boston, Massachusetts 02115, USA.

\* To whom correspondence should be addressed: Dept. of Microbiology and Molecular Genetics, Harvard Medical School, 633 Armenise Building, 200 Longwood Ave, Boston, MA 02115; Tel: 617-432-5488 ; Fax: 617-738-7664;  
E-mail: [suzanne\\_walker@hms.harvard.edu](mailto:suzanne_walker@hms.harvard.edu),

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## ABSTRACT

Wall teichoic acids are cell wall polymers that maintain the integrity of the cellular envelope and contribute to the virulence of *Staphylococcus aureus*. Despite the central role of wall teichoic acid in *S. aureus* virulence, details concerning the biosynthetic pathway of the predominant wall teichoic acid polymer are lacking and have relied on a presumed similarity to the putative polyribitol phosphate wall teichoic acid pathway in *Bacillus subtilis*. Using a high resolution polyacrylamide gel electrophoresis separation method for analysis of wall teichoic acid extracted from gene deletion mutants, a revised assembly pathway for the late stage ribitol phosphate utilizing enzymes is proposed. Complementation studies show that a putative ribitol phosphate polymerase, TarL, catalyzes both the addition of the priming ribitol phosphate onto the linkage unit and the subsequent polymerization of the polyribitol chain. It is shown that the putative ribitol primase, TarK, is also a bifunctional enzyme that catalyzes both ribitol phosphate priming and polymerization. TarK directs the synthesis of a second, electrophoretically distinct polyribitol containing teichoic acid that we designate K-WTA. The biosynthesis of K-WTA in *S. aureus* strain NCTC8325 is absolutely dependent upon a functional accessory gene regulator (*agr*) locus. The demonstration of regulated wall teichoic acid biosynthesis has implications for cell envelope remodeling in relation to *S. aureus* adhesion and pathogenesis.

## INTRODUCTION

Wall teichoic acids (WTA) are anionic, carbohydrate-based polymers that are covalently attached to the peptidoglycan matrix of many Gram-positive bacteria (32, 41, 44). In *Bacillus subtilis*, WTA accounts for 30-60% of the total cell wall mass and has been implicated in a number of roles critical to maintaining the overall integrity of the cell envelope (21). The loss of cell-surface charge balance, tensile strength, rigidity, porosity, proper cell morphology, and misregulation of autolysins are all associated with mutations in WTA related genes (32). In addition to a structural role, the WTA polymer itself may also serve as a phosphate reservoir for transition to growth in phosphate depleted medium and in cation ( $Mg^{2+}/Ca^{2+}$ ) assimilation/homeostasis (12).

While much is known about the structure and function of WTA in rod-shaped bacteria, comparatively little is known about the role of WTA in coccoid bacteria. In contrast to *B. subtilis* (10), *Staphylococcus aureus* lacking WTA is not significantly impaired in growth or fitness (11, 25, 45). *S. aureus* WTA has been shown, however, to play an essential role in adhesion to endothelial and epithelial tissues, and to be critical for colonization in multiple infection models (2, 45, 46). Since adhesion is a key step in infection, WTA can be considered a quintessential virulence factor, making it a potential target for antimicrobial intervention. To exploit the WTA pathway as a novel drug target in *S. aureus*, it is necessary to define the activity of the enzymes in the pathway and to elucidate the mechanism of assembly.

The majority of *S. aureus* strains contain poly ribitol phosphate (RboP) WTA, for which the biosynthetic pathway has been modeled using the prototype polyRbo WTA producing strain, *B. subtilis* strain W23 (28). DELETED The proposed pathway has been

1 accepted in *S. aureus* despite major differences in WTA gene organization between *S.*  
2 *aureus* and *B. subtilis* strain W23 (38). There are at least two separate polycistronic WTA  
3 gene clusters (in addition to the monocistronic *tarO* gene) in the *S. aureus* genome,  
4 whereas the WTA genes in *B. subtilis* W23 are in a single, divergently transcribed locus.  
5 In particular, the proposed poly RboP WTA assembly model invokes two sequentially  
6 acting enzymes, a RboP primase (TarK) and a RboP polymerase (TarL). Qian *et. al.*  
7 noted that for this model to be correct in *S. aureus*, two enzymes (TarK and TarL) with  
8 more than 80% sequence identity would have to catalyze distinct reactions, namely the  
9 RboP priming and RboP polymerization. Since high sequence similarity is not  
10 uncommon among WTA polyol biosynthetic enzymes and a common donor (CDP-Rbo)  
11 is used by both enzymes, we sought to establish the pathway by experimentally  
12 addressing whether *S. aureus* has a unique three enzyme pathway where TarK and TarL  
13 are functionally redundant or if indeed they have evolved unique activities despite limited  
14 primary sequence divergence.

## 16 MATERIALS AND METHODS

17 ***Strains and growth conditions*** — All *S. aureus* strains used are derivatives of the  
18 sequenced NCTC8325 reference strain (18). Plasmids were constructed in *Escherichia*  
19 *coli* Novablue (Novagen) cells, and introduced into the restriction negative *S. aureus*  
20 strain RN4220 by electroporation (40). Plasmids were purified by pre-treating cells with  
21 lysostaphin (50 g/mL, 10 min) prior to isolation using a standard plasmid miniprep  
22 protocol (Qiagen). Methylated plasmids were then introduced into restriction positive  
23 wildtype strains by electroporation. *S. aureus* was grown in either tryptic soy broth (TSB)

1 or Luria-Bertani (LB) medium at 37 °C unless otherwise noted. Antibiotic markers were  
2 selected with erythromycin (Em; 10 µg/mL), tetracycline (Tc; 2.5 µg/mL), and  
3 chloramphenicol (Cm; single copy integrated into genome 5 µg/mL, plasmid 10 µg/mL).  
4 Bacterial strains used are listed in Table 1.

5 ***Construction of S. aureus WTA gene deletion strains*** — Genes were deleted  
6 using a modified protocol for the pKOR1 *E. coli*/*S. aureus* shuttle vector (4). The  
7 pKOR1 plasmid has a temperature sensitive *S. aureus* replicon, and a Tc inducible  
8 antisense *secY* cassette for counterselection. In-frame gene deletion cassettes with  
9 flanking homology arms were constructed by overlap assembly PCR, using primer pairs  
10 (P1,P2 and P3,P4) to amplify ~1 kb upstream/downstream of the targeted gene.  
11 Fragments were assembled by a second round of PCR to obtain a deletion cassette  
12 containing the first and last 90 base pairs of the targeted gene. The cassette was digested  
13 (*Apa*I), and ligated into restricted pKOR1 vector (*Apa*I/*Eco*RV) to yield integration  
14 plasmids pMS17/18 (Table 1). A marked Tc<sup>r</sup> deletion cassette was constructed by  
15 ligation of *tetL* (amplified from pCL25, tetL-for, tetL-rev) into the P1,P4 *tarL* PCR insert  
16 (*Bst*BI/*Mlu*I) to give pMS21. Primers and gene deletion vectors are listed in Table 2.

17 Plasmids were electroporated into *S. aureus* RN4220 and plated on TSB agar (10  
18 g/mL Cm, 30 °C). Plasmid containing clones were then inoculated into 50 mL of pre-  
19 warmed TSB culture and grown at 42 °C for 12-15 generations (~4 hrs) without selection  
20 to reduce plasmid copy number and favor integration. Cm (5 g/mL) was then added,  
21 and growth continued overnight. Cultures were streaked to single colonies (42 °C; 5  
22 g/mL Cm), and clones were screened for plasmid integration by PCR using P1-P4 to  
23 verify co-integrates. Clones from which wild-type and mutant alleles were amplified at

1 approximately equal intensities were resolved by overnight growth in TSB at 30 °C,  
2 followed by pKOR1 vector counterselection on TSA with anhydrotetracycline (1 g/mL).  
3 Gene deletion in Cm<sup>s</sup> clones was confirmed by PCR analysis using primers that anneal  
4 outside the particular P1-P4 deletion cassette.

5 **Plasmid Construction** — The low-copy (4-5 copies/cell), cadmium-inducible  
6 complementation vector pMS74 was constructed by ligating the pI258 replicon from  
7 pRN8298 into the parent pCN59 vector (7) using the restriction sites *NarI/ApaI*. The  
8 *tarK* gene was then amplified (TarK<sub>pen</sub>-for, TarK<sub>pen</sub>-rev) to incorporate flanking  
9 restriction sites and ligated into pMS74 (*SaII/AseI*). The accessory gene regulator (*agr*)  
10 complementation vector was made by PCR amplification of the wildtype *agrA* allele  
11 along with P1 promoter and terminator [*agrA*-F, *agrA*-R (42)] and subsequently ligated  
12 into pCN33 using *EcoRI/SphI*. The promoter probe plasmids pXen- P<sub>*tarI'*J'K</sub> and -P<sub>*tarI*JL</sub>  
13 *luxABCDE* were made by ligating the intergenic DNA sequences directly upstream of the  
14 respective start codon for *tarI'* (528 base pairs; ProtarI'<sub>J'K</sub>-for, ProtarI'<sub>J'K</sub>-rev) and *tarI*  
15 (279 base pairs, ProtarI<sub>JL</sub>-for, ProtarI<sub>JL</sub>-rev) into pXen-1 (*EcoRI/BamHI*). The plasmid  
16 pMS80 was assembled by cloning the *tarO* locus (TarO-F, TarO-R) into pLI50 (29).

17 Site-specific integration plasmids based on the bacteriophage L54a *attP*  
18 recombination system were constructed by ligation of the *int-attP* fragment (*ClaI/BamHI*)  
19 of pCL55 into pCL25 (*ClaI/BglII*) (29). The *tetL* marker (partial digest *SpeI/ClaI*) was  
20 then replaced with the *ermC* marker from pCN59 (*AvrII/ClaI*) by ligation to make  
21 pCL25int. An IPTG-inducible promoter (P<sub>spank</sub>) was installed by ligation of the P<sub>spank</sub>-  
22 MCS-P<sub>pen</sub>*lacI* cassette of pDR110 (D. Rudner, Harvard Medical School) into pCL25int  
23 with *EcoRI/BamHI* to give pCL25intP<sub>spank</sub>. A version containing the strong, constitutive

P<sub>Pen</sub> promoter was constructed by PCR-amplification of this module from pDR110 (P<sub>penfor</sub>-P<sub>penrev</sub>), and then swapping the P<sub>Pen</sub> promoter for the constitutive P<sub>blaZ</sub> promoter of pCN68 (7) using *SphI/SalI* restriction sites. In our hands, particularly if used on a multi-copy plasmid, the P<sub>Pen</sub> promoter was considerably more stable and less prone to rearrangement than the P<sub>blaZ</sub> promoter of pCN68. Genes were cloned into the modified vector using amplified PCR products containing *EcoRI/SalI* and *AseI* restriction sites. The P<sub>Pen</sub>-MCS-gene-*blaZ* transcriptional terminator (TT) cassettes were then excised from pCN68 vector backbone (*SphI/SfoI*) and ligated into the MCS of pCL25int (partial *SphI/SmaI*) to make pCL25intP<sub>Pen</sub>. Integration plasmids were electroporated into RN4220, and then moved by transduction into RN450 (see below). Plasmids and primers are listed in Table 2.

**WTA extraction** — WTA was isolated from 20 mL culture of *S. aureus* stationary phase cells grown in TSB overnight at 37 °C. Cells were collected by centrifugation (2000 x g, 10 min), washed once with 30 mL of Buffer 1 [50 mM 2-(*N*-morpholine)-ethane sulphonic acid (MES), pH=6.5], and resuspended in 30 mL of Buffer 2 [4 % w/v sodium dodecyl sulfate (SDS), 50 mM MES, pH=6.5]. Samples were placed in a boiling water bath for 1 hr, and then the cells were collected by centrifugation (10,000 x g, 10 min). The pellet was resuspended in Buffer 2, transferred to a 2 mL microcentrifuge tube, and sedimented (14,000 x g, 10 min). The pellet was next washed once with Buffer 2, once with Buffer 3 (2 % NaCl, 50 mM MES, pH 6.5), and finally with Buffer 1. After the last wash, samples were treated with proteinase K [20 mM Tris (HCl), pH=8.0, 0.5 % w/v SDS, 20 g of proteinase K in 1 mL] and incubated at 50 °C for ~4 hr. Following digestion, samples were washed once with Buffer 3, followed by at least three washes

1 with dH<sub>2</sub>O to remove SDS. Samples were thoroughly resuspended in 1 mL of 0.1 M  
2 NaOH and shaken at room temperature for 16 hr to hydrolyze WTA (13). Insoluble cell  
3 wall debris was removed by centrifugation (14,000 x g, 10 min), and the supernatant  
4 containing the hydrolyzed WTA was either directly analyzed by polyacrylamide gel  
5 electrophoresis (PAGE) or further processed for long-term storage. Samples were  
6 neutralized with 0.1 M acetic acid, extensively dialyzed against dH<sub>2</sub>O (MWCO= 1 kDa),  
7 and then lyophilized to a white powder. Approximately 500 µg of crude WTA was  
8 typically isolated from a 20 mL culture.

9 **WTA PAGE analysis** — The WTA PAGE protocol of Pollack and Neuhaus (37)  
10 used for *B. subtilis* polyGroP WTA analysis was modified to further improve *S. aureus*  
11 polyRboP WTA resolution. The Biorad Protean II xi electrophoresis cell was used for  
12 separation (20 cm x 16 cm x 0.75 mm). The separating gel [20 % T (total acrylamide), 6  
13 % C (percentage of T that is the cross-linker *bis*-acrylamide)] was cast by mixing 20 mL  
14 of Tris-HCl buffer [3 M Tris-HCl, pH= 8.5] with 40 mL of acrylamide stock solution (30  
15 % T, 6 % C). The solution was polymerized using 600 µL of 10 % ammonium persulfate  
16 along with 60 µL of tetramethylethylenediamine (TMED). The stacking gel (3 % T, 0.26  
17 % C) was ~1 cm long and was cast using a mixture of 1 mL acrylamide stock solution  
18 (30 % T, 0.26 % C), 3 mL Tris-HCl buffer [3 M Tris(HCl), pH 8.5], and 6 mL of dH<sub>2</sub>O.  
19 The stacking gel was polymerized with 100 µL of 10 % ammonium persulfate and 10 µL  
20 of TMED. WTA samples were diluted 1:3 in loading buffer [50 % glycerol in running  
21 buffer with a trace of bromophenol blue] to a final volume no greater than 10 µL.  
22 Typically, ~200 ng of crude WTA sample was loaded for each sample. Gels were  
23 developed at 4 °C for ~18 hr under constant current (80 mA with two gels) using a Tris-



Tricine running buffer [0.1 M Tris base, 0.1 M Tricine, pH 8.2 from (39)]. WTA bands were visualized using the reported alcian blue/silver staining protocol (37).

**Luciferase Promoter Reporter Assay** — Overnight cultures of RN450 carrying the pXen plasmid with promoter fragments were diluted 1:200 into fresh TSB (10 g/mL Cm) and incubated at 37 °C with shaking (200 rpm). Aliquots were withdrawn at 30 min intervals, and cells pelleted by centrifugation (5,000 x g, 2 min). Cells were resuspended in PBS buffer, transferred to a 96-well microplate, and the OD<sub>532</sub> (Perkin Elmer ITS 7000) and luminescence (Perkin Elmer MicroBeta TriLux) was measured to estimate promoter activity.

**Phage Infection and transduction** — Plaque forming units (PFU) of various hosts strains were determined by mixing phage (~500 PFU) with exponentially dividing cells (OD<sub>600nm</sub>=0.5) grown in TSB-Ca [(TSB with CaCl<sub>2</sub> (200 g/mL)]. Mixtures were incubated (30 °C, 20 min), suspended in TSB-Ca top agar (0.5% agar), and then poured over TSB-Ca agar plates (1.5% agar). Plaques were counted after 16 hr of growth at 30 °C.

Donor lysates were obtained by plating as described above except the generalized transducing phage ø11 was used at a concentration to obtain confluent top agar phage lawns. Phage were eluted by the addition of TSB and gentle shaking overnight at room temperature. Lysates were filter sterilized (0.45 M), and checked for lack of viable donor bacteria by plating. Recipient bacteria were grown in TSB to mid-logarithmic growth (2 mL cultures), at which point CaCl<sub>2</sub> was added to 0.5 mM. Donor lysate was added, and the tubes incubated at 30°C (15 min). For phage sensitive recipient strains, mixtures were pelleted and washed three times with TSB containing 20 mM citrate

1 before addition of top agar. Otherwise, 10 mL of TSB top agar (0.5% agar) was directly  
2 added, and the mixture poured over TSB agar plates. Inducing amounts of antibiotics  
3 (0.05 g/mL Em or Tc) were included in the top agar to facilitate phenotypic expression  
4 during incubation at 37 °C (1 hr). Plates were then overlayed with 15 mL of TSB top  
5 agar containing antibiotic at a selective concentration (12.5 g/mL Tc, 10 g/mL Em).  
6 Transductants were scored after 48 hours of incubation at 37 °C.

7 To transduce markers into the *tarO* background (J122), *tarO* was provided on a  
8 plasmid (pMS80) to temporarily restore phage WTA receptor. After transduction, the  
9 plasmid was cured by protoplast regeneration to restore the *tarO* genotype (20).

10  
11 ***Phenotypic analysis of agr Expression*** — Because of the inherent instability of  
12 the accessory gene regulator (*agr*) locus (42), the expression of *agr* was explicitly  
13 determined for each strain used in this study. The production of  $\alpha$ -hemolysin was scored  
14 on TSA 5% sheep blood agar plates (BD, TSAII™) as described (1). Extracellular  
15 protein (EC) fractions were obtained from overnight stationary phase cultures grown in  
16 LB by pelleting to remove bacteria, and then filter sterilized (0.45  $\mu$ m). Protein was  
17 concentrated by precipitation using a modified sodium deoxycholate-trichloroacetic acid  
18 protocol (5), and then separated by SDS-PAGE (12% acrylamide). Protein samples were  
19 either stained for total protein content with Coomassie blue or blotted to nitrocellulose  
20 membranes. An amount of EC protein corresponding to 1 mL of culture (OD<sub>600nm</sub>  
21 adjusted to 1) was loaded for each sample for total protein staining, while ~5 g of EC  
22 protein per sample was loaded for gels to be probed by western blotting. Membranes  
23 were developed by blocking overnight in TBST with BSA (5%), and then probed with

1 sheep polyclonal anti -hemolysin-HRP conjugate (Abcam ab15949) antibody following  
2 the manufacturer's standard protocol.

## 4 RESULTS

5 ***The tarL gene is an essential WTA gene while tarI'J'K are dispensable*** — The  
6 WTA gene cluster of all *S. aureus* genomes sequenced to date contains two ~3.4 kb gene  
7 clusters (*tarI'J'K* and *tarIJJL*) with over 75% DNA sequence identity between them (Fig.  
8 1A) (38). These clusters are putatively involved in the biosynthesis of the polyRboP  
9 repeat, through the formation of CDP-Rbo (*tarI'J'/tarIJ*) and subsequent polymerization  
10 (RboP priming *tarK*/RboP polymerization *tarL*) onto the linkage unit acceptor. To  
11 address whether these clusters have distinct activities or are simply duplicated genes, in-  
12 frame gene deletion vectors were constructed using the temperature sensitive replication  
13 vector pKOR1 (4). While numerous excisants derived from *tarK* or *tarI'J'* co-integrates  
14 retained the *tarK* and *tarI'J'* mutant allele, respectively, *tarL* and *tarIJ* co-integrates  
15 that were resolved using the same procedure consistently failed to yield deletion mutants.  
16 This suggests that the *tarI'J'K* cluster is dispensable, while the *tarIJJL* cluster is essential  
17 in *S. aureus* RN4220.

18 Regulated ectopic expression coupled with deletion of the wildtype allele is an  
19 established method for gene essentiality testing in *S. aureus* (14). The Spank system was  
20 chosen since the basal level is sufficiently low to generate conditionally lethal phenotypes  
21 in *S. aureus* (23, 48). To confirm that the *tarL* gene is indeed essential in wildtype cells  
22 and that lack of deletion mutants was not attributable to polar affects, a *tarL* depletion  
23 strain (JT16) was constructed by placing a second chromosomal copy of *tarL* in the

1 phage L54a integration site under the control of the IPTG-inducible P<sub>spank</sub> promoter. The  
2 native *tarL* gene was then deleted using the standard pKOR1 procedure except IPTG  
3 inducer was included in the media during the co-integrant resolution step. Growth  
4 analysis showed an absolute dependence on IPTG and hence *tarL* expression, indicating  
5 TarL is an essential enzyme in the WTA pathway (Fig. 1B). When the first gene in the  
6 pathway (*tarO*) is also deleted in the same background (JT22), the *tarL* gene is no longer  
7 necessary for growth. This conditional dispensability is consistent with a WTA gene  
8 deletion study by D'Elia *et al* who observed a similar pattern with WTA linkage unit  
9 genes (11). However, the nonessential nature of *tarK*, an enzyme whose activity is  
10 predicted to occur prior to *tarL*, contradicts the proposed RboP primase-RboP polymerase  
11 model (11).

12 ***S. aureus synthesizes two distinct polyRboP WTA polymers*** — Since it was  
13 possible that the RboP primase-RboP polymerase model could still be correct if the  
14 putative truncated WTA of *tarK* (and possibly *tarI'J'*) deletion strain is somehow  
15 nontoxic in comparison to the predicted RboP-primed WTA precursor of the *tarL* strain,  
16 the presence of mature polyRboP WTA was probed using a panel of bacteriophages.  
17 WTA is the primary receptor for the majority of Gram-positive bacteriophages, including  
18 the polyRboP-WTA specific phages of *S. aureus* (3). Thus, the ability of a panel of  
19 phages to infect the mutant strains was measured to probe for structural and/or  
20 compositional WTA alterations due to deletion of genes within the *tarI'J'K* cluster.  
21 However, all three phages tested formed plaques with efficiencies comparable to  
22 wildtype (Fig. 2A). Collectively, the data suggests that both TarK and TarL can catalyze

1 polyRboP TA chain polymerization using a common pool of CDP-ribitol donor (*tarI'J'*  
2 and *tarIJ*).

3 To determine whether the clusters were simply duplicated or if there were more  
4 subtle differences between the strains that do not alter susceptibility to phage infection.  
5 WTA was directly analyzed by extraction and separation by PAGE. Numerous standard  
6 extraction conditions were tested, including acid catalyzed (5% trichloroacetic acid), base  
7 catalyzed (0.1 N NaOH), and nucleophilic mediated WTA cleavage at neutral pH using  
8 an aqueous solution of saturated lithium thiocyanate. While all methods produced  
9 comparable WTA profiles (data not shown), base mediated cleavage performed  
10 according to Endl *et al.* consistently provided the best WTA yield (13). The PAGE  
11 protocol that produced well-spaced, single band resolution for polyGroP WTA of *B.*  
12 *subtilis* 168 (37) did not give satisfactory results in our hands when using *S. aureus*  
13 polyRboP WTA. Accordingly, the PAGE protocol was systematically adjusted (see  
14 Materials and Methods) until acceptable resolution was attained (Fig. 2B). Unlike the  
15 WTA depleted *tarO* control sample, the *tarI'J'* strain yielded both a similar WTA  
16 quantity and profile in comparison to the wildtype RN4220 strain. The *tarK* WTA  
17 chains actually lengthened by 7-10 units (Fig. 2A, lane 4), in contrast to truncated WTA  
18 chains as would be expected in the TarK RboP primase model. The shift to a longer  
19 average repeat was accompanied by a decrease in WTA heterogeneity, with a series of  
20 single repeat-spaced bands seen in the wildtype sample disappearing (Fig. 2B, arrows).  
21 The conversion of heterogeneous, short WTA (wildtype) to long, homogeneous WTA  
22 ( *tarK*) could be controlled stepwise by placing TarK under the control of a Cd<sup>2+</sup>  
23 promoter (Fig. 2B, lanes 5 and 6). The banding pattern indicates *S. aureus* makes two

1 distinct WTA polymers, a primary TarL-directed (L-WTA) and a secondary TarK-  
2 directed (K-WTA) polymer. The retention of K-WTA in the *tarI'J'* strain suggests  
3 *tarIJ* can provide the CDP-ribitol donor needed for biosynthesis of both TA polymers.

4 ***The tarI'J'K promoter is significantly weaker than the tarIJL promoter*** — The  
5 apparent inability to obtain cross-complementation with TarK and TarL despite both  
6 enzymes seemingly making a polyRbo-WTA raised the possibility of differential  
7 expression from their respective native promoters. The putative upstream promoter  
8 regions from the *tarI'J'K* (528 bp) and *tarIJL* (279 bp) clusters were cloned into the  
9 luciferase based promoter probe plasmid pXen1 (17). Both promoters drove expression  
10 of the *luxABCDE* cassette in comparison to the empty vector background control (Fig.  
11 3A). The *tarIJL* promoter, however, produced ~30-fold more luminescence than the  
12 *tarI'J'K* promoter and suggests L-WTA is the predominant form of WTA in *S. aureus*.

13 ***TarL and TarK of S. aureus are bifunctional enzymes*** — The substantial  
14 difference in promoter strengths between the *tarI'J'K* and *tarIJL* clusters suggested TarK  
15 may be able to complement TarL if overexpressed from a strong constitutive promoter.  
16 To explore this possibility, various WTA genes were placed under control of the Gram-  
17 positive P<sub>pen</sub> promoter and stably integrated into the chromosome of recipient strains. A  
18 *tarL::tetL* allele phage donor strain (JT16) was constructed to test complementation by  
19 scoring transduction efficiency into recipient strains. The wildtype strain RN450 was  
20 used instead of RN4220 since this strain has been mutagenized to accept foreign DNA  
21 and may harbor unmapped mutations (27). In addition to overexpressing a given gene,  
22 the chromosomal *tarK* gene of each recipient strain was deleted so that the presence of  
23 Tc<sup>r</sup> transductants signaled that the given gene alone could complement TarL *in trans*.

1 Otherwise, the *tarL::tetL* allele would be lethal as in the wildtype recipient (Fig. 3B).  
2 Indeed, TarK from *S. aureus* could replace TarL provided it was sufficiently expressed.  
3 The transduction mediated complementation experiment was also performed with  
4 recipients harboring integrated copies of TarK and/or TarL from *B. subtilis* W23 (TarK<sup>Bs</sup>  
5 TarL<sup>Bs</sup>, and TarKL<sup>Bs</sup>). While TarK<sup>Bs</sup> and TarL<sup>Bs</sup> alone could not complement,  
6 transductants were obtained at efficiencies comparable to the *S. aureus* TarL positive  
7 control recipient when both *B. subtilis* genes (TarKL<sup>Bs</sup>) were supplied simultaneously.  
8 Thus, both TarL and TarK from *S. aureus* are bifunctional enzymes with activity (*i.e.*  
9 RboP priming and polymerization) equivalent to the two monofunctional *B. subtilis* W23  
10 RboP enzymes, TarK<sup>Bs</sup> and TarL<sup>Bs</sup>.

11 To confirm restoration of WTA biosynthesis in the complemented strains, WTA  
12 was extracted and analyzed by PAGE (Fig. 3C). All strains synthesized high molecular  
13 weight WTA polymer, including the *S. aureus*-*B. subtilis* W23 hybrid pathway. The  
14 WTA profile of the  $\phi$ 11 lysogenized strain (JT200) was identical to wildtype (RN450),  
15 indicating phage integration does not influence WTA composition. The WTA extracted  
16 from the strain overexpressing TarL (JT213) was nearly identical to both the lysogenized  
17 wildtype control strain (JT200) and to the *tarK* (JT207) strain (see below), forming long,  
18 homogeneous polymers of L-WTA with little to no K-WTA. In comparison,  
19 overexpression of TarK (JT214) without any TarL present resulted in a shorter average  
20 polymer length by ~10 repeats. While K-WTA is the predominant species as would be  
21 expected, a series of intervening bands of diminished intensity was clearly discernible.  
22 The banding pattern of the hybrid *S. aureus*-*B. subtilis* W23 WTA pathway strain (JT215)

1 was less complex, yielding a homogeneous ladder comprised of polymers that migrated a  
2 distance identical to *S. aureus* L-WTA.

3 ***The accessory gene regulator (agr) influences WTA length through TarK —***  
4 The WTA profile of the RN450 strains (Fig. 3C, lanes 1 and 2) is similar to  
5 RN4220( *tarK*) (Fig. 2B, lane 4) in both length and lack of K-WTA heterogeneity.  
6 Further, deletion of *tarK* from RN450 does not lead to a lengthening of WTA (Fig. 3C,  
7 lane 2 versus 3) as observed in RN4220, hinting at differential *tarK* regulation between  
8 the two strains. This is surprising considering RN4220 was directly derived from the  
9 parent RN450 strain (27). While no known RN4220 specific mutations map to WTA  
10 related genes, RN4220 is partially *agr*<sup>−</sup> (accessory gene regulator) due to a frameshift  
11 mutation in *agrA* (42). The *agr* system is two component quorum sensing network that  
12 attenuates expression of numerous virulence-related cell surface features (8, 34), and was  
13 thus a potential cause for the observed strain dependent expression of K-WTA.

14 The *agr* locus is genetically unstable, particularly during cultivation outside the  
15 host (42), and it was therefore necessary to explicitly link the *agr* phenotype to the WTA  
16 profile using paired isogenic strains. The *agr::tetM* null allele was transduced into three  
17 commonly used NCTC8325 strains, JT200 [RN450(Ø11)], SA113 [NCTC8325  
18 (Ø11,Ø12,Ø13)], and SH1000 (RN450 *rsbU*<sup>+</sup>). The *agr*<sup>+</sup> phenotype was evaluated by  
19 comparison to the respective *agr::tetM* transductant using the following criteria : 1)  
20 positive  $\alpha$ -hemolysin (RNAIII) typing on TSA-blood agar, 2) high level of extracellular  
21 (EC) protein production, and 3) secretion of  $\alpha$ -hemolysin. The RN450 (JT200) and  
22 SH1000 strains used in this study were clearly *agr*<sup>+</sup>, producing RNAIII encoded  $\alpha$ -  
23 hemolysin (the enhanced zone of clearing within the inner halo at the cross streak



intersection due to synergistic hemolysis with RN4220 secreted  $\alpha$ -hemolysin (1); Fig. 4A) and a comparatively large quantity of extracellular proteins (Fig. 4B), of which a higher percentage was  $\alpha$ -hemolysin (upregulated by *agr*; Fig. 4C). In comparison, SA113 was nonhemolytic and presumably *agr*<sup>−</sup>. The WTA profiles of the strains corresponded without exception to the *agr* phenotype (Fig. 5), *i.e.* long, homogeneous L-WTA polymers in *agr*<sup>+</sup> strains and short, heterogeneous polymers in *agr*<sup>−</sup> strains. The relationship is independent of the expression level of the alternative sigma factor ( $\sigma^{B}$ ) as SH1000 behaved analogously to parent *rsbU*<sup>−</sup> strains. The effect of deleting *agr* on WTA composition is not seen in a *tarK* background (JT221), indicating *agr* specifically modulates WTA through *tarK* and the chain shortening is not due to a general pleiotropic mechanism such as altering growth rate (Fig. 5). Further, complementation of the *agr* defect in RN4220 (JT38) represses K-WTA synthesis and lengthens WTA to match the parent RN450 strain. The WTA expression profile in RN4220 is therefore the result of a single lesion in the *agr* locus, and indicates the *agr* global regulatory system represses synthesis of K-WTA.

## DISCUSSION

It has been suggested that the genes *tarK* and *tarL* from *S. aureus* each have a unique enzymatic activity, namely the RboP priming (TarK) of the linkage unit and the subsequent RboP polymerization (TarL) to complete the TA chain (11). This model was based on the proposed *B. subtilis* W23 polyRboP pathway (28), though there is limited experimental evidence concerning the enzymes involved in RboP addition from either bacterial strain. The validity of extending the *B. subtilis* W23 two-enzyme RboP

1 primase-polymerase model to *S. aureus* has been questioned based on computational  
2 analysis of the gene clusters, which revealed not only differing genetic organization, but  
3 also an apparent 3-gene duplication specific to *S. aureus* [*tarI'J'K* and *tarLJL*; Fig. 1A]  
4 (38). For the two enzyme RboP primase-polymerase model to hold true in *S. aureus*, two  
5 proteins TarK and TarL (80% identity, 90% similar) would have to catalyze distinct  
6 reactions. Qian *et al.* suggested different activities were possible given the sequence  
7 divergence between the N-termini of TarK and TarL (38). They also suggested a second  
8 possibility that the two clusters may actually be functionally redundant, which would  
9 mean there is no RboP priming enzyme in the pathway. This study suggests a third  
10 scenario, in which both TarL and TarK are indeed bifunctional RboP priming/RboP  
11 polymerizing WTA enzymes but yet remain functionally nonredundant. We propose  
12 TarL and TarK direct the synthesis of two distinct cell wall WTA polymers (a primary L-  
13 WTA and a secondary K-WTA, respectively) in a revised polyRboP WTA pathway (Fig.  
14 6). We further suggest the sequence divergence in the N-termini between TarK and TarL  
15 actually reflects different linkage unit substrate specificity and not a shift between RboP  
16 priming/polymerizing.

17 A regulated ectopic expression system was used to show TarL is an essential gene  
18 (Fig. 1B), but that *tarK* is viable. The *tarK* strain makes full length WTA RboP  
19 polymer, thus ruling out a dedicated RboP primase activity *in vivo* for TarK of *S. aureus*.  
20 Secondly, the WTA heterogeneity as analyzed by high resolution PAGE directly  
21 corresponds to expression of TarK. Strains only expressing TarL are homogeneous with  
22 respect to extractable WTA, whereas strains expressing both enzymes have a second set  
23 of intervening bands that is dependent upon TarK. The unique electrophoretic mobility

1 suggests K-WTA has a different structure than L-WTA, and that *S. aureus* NCTC8325  
2 has at least two WTA polymer types. While the structure of the newly identified K-WTA  
3 is not known, all phages tested infected equally well whether K-WTA was produced at  
4 basal levels (RN4220), deleted (JT18), or overexpressed in the presence (JT209) or  
5 absence of TarL (JT214) (Fig. 2A and data not shown). Since polyRboP WTA is the  
6 receptor for phage attachment (3), and strains expressing only *tarK* remain susceptible,  
7 K-WTA is equivalent to L-WTA with regards to phage binding and is therefore likely  
8 composed of RboP repeats. This is consistent with the retention of K-WTA when the  
9 *tarI'J'* genes are deleted as *tarIJ* (characterized CDP-Rbo synthesizing enzymes (36))  
10 can supply the common donor substrate pool for both L-WTA and K-WTA. The  
11 difference between the two structures may stem from a polyRboP chain modification or  
12 from differences within the linkage unit. The two known WTA modifications in *S.*  
13 *aureus* are glycosylation with D-GlcNAc and esterification with D-alanine residues (32).  
14 However, deletion of the two putative glycosyl transferases located within the WTA  
15 clusters (SAOUHSC\_00644 and SAOUHSC\_00228) does not affect the amount of K-  
16 WTA in RN4220 (Meredith, T.C, Swoboda, J.G., and Walker, S.; unpublished), and D-  
17 alanine TA esters are highly labile under the basic conditions used for extraction (31).  
18 Alkaline hydrolysis of WTA occurs between C-4 of ManNAc and the proximal GroP  
19 within the linkage unit (26). Barring the existence of unknown WTA modifications  
20 and/or an alternative connection to the linkage unit disaccharide core, this would localize  
21 the structural difference between K-WTA and L-WTA to the linkage unit region distal to  
22 ManNAc but prior to the polyRboP repeat. A potential candidate is the number of GroP

1 units, as both (GroP)<sub>2</sub>- and (GroP)<sub>3</sub>-ManNAc-GlcNAc-PP-Und have been shown to serve  
2 equally well as acceptors for RboP units in *S. aureus* H membranes (47).

3 Transduction efficiency analysis yielded clear results, showing TarK could indeed  
4 complement TarL if provided at levels much higher than produced from its relatively  
5 weak endogenous promoter in RN450 (Fig. 3A and 3B). The WTA profile when only  
6 TarK is expressed from an unregulated strong promoter (JT214) not only increases the  
7 amount of K-WTA as would be anticipated (shortened chain length), but also produces  
8 heterogeneity despite TarL being absent (Fig. 3C). This suggests TarK can synthesize L-  
9 WTA when overexpressed from a strong promoter, albeit much less efficiently than K-  
10 WTA. The accumulation of WTA intermediates is lethal in *S. aureus* [see D'Elia *et al.*  
11 for discussion (11)]. The need for high level TarK expression to support viability in  
12 *tarL* background likely reflects the need to utilize an alternative linkage unit acceptor  
13 substrate to alleviate the build-up of WTA intermediates.

14 The *S. aureus* TarK and TarL results raised the possibility that the *B. subtilis* W23  
15 model may also be incorrect. However, the heterologous complementation results  
16 support a two enzyme RboP priming-RboP polymerization pathway in *B. subtilis* W23, as  
17 proposed (28), since both TarK<sup>Bs</sup> and TarL<sup>Bs</sup> enzymes are needed to replace the  
18 bifunctional *S. aureus* TarL enzyme (Fig. 5 and 6). The hybrid WTA pathway strain  
19 makes a single WTA species, suggesting the putative K-WTA linkage unit acceptor in  
20 not recognized by TarKL<sup>Bs</sup>.

21 Both TarK and TarL of *S. aureus* are bifunctional RboP priming and  
22 polymerizing enzymes, implying active site plasticity. While the CDP-Rbo donor is the  
23 same, each activity requires the utilization of a primary hydroxyl acceptor with an -

1 carbon secondary alcohol differing in stereochemistry (Fig. 6B). Active site plasticity  
2 may explain why lipoteichoic acid (LTA), a GroP polyol with differing stereochemistry  
3 than GroP of the WTA linkage unit (32), can serve as an acceptor *in vitro* but does not  
4 occur *in vivo* due to LTA termini accessibility (15, 16).

5       The biological significance of having the genetic determinant for K-WTA in  
6 addition to the primary L-WTA system is at present unknown. However, the observation  
7 that all *S. aureus* strains have the K-WTA cluster separated from the main WTA cluster  
8 by an intergenic gap of variable length suggests a conserved role (38). PAGE analysis  
9 confirmed an increase in average polyRboP chain length by approximately 7-10 repeat  
10 units when either *tarK* is deleted or when the *agr* system is functional (Fig. 2B and 5).  
11 The average chain length of polyRboP WTA in RN450 as determined by compositional  
12 analysis has previously been estimated to be ~22 RboP repeats (24), indicating the *agr*  
13 system can change the average length of WTA by up to 50%. This alone would be  
14 expected to significantly influence both the bacterial surface and the degree of surface  
15 accessibility for intracellular contacts. While details concerning the mechanism of *agr*  
16 regulation on K-WTA are still needed, a clear *agr* and *tarK*-dependent effect on WTA  
17 length and composition in *S. aureus* NCTC8325 has been demonstrated (Fig. 5). The  
18 quorum sensing *agr* system is a global regulator of numerous virulence determinants that  
19 is most active during the transition from exponential to postexponential growth (8, 34).  
20 The *agr* locus generally upregulates expression of exoproteins (including  $\alpha$ -hemolysin)  
21 while repressing the synthesis of cell-surface adhesins. The net effect is to transition the  
22 bacterial surface from a “pro-adhesion” state to a “low-adhesion” state in order to  
23 facilitate dissemination once a local cell density has been reached (8, 34). Given the role

1 of WTA in adhesion to mammalian cells (45, 46), it is possible to speculate that *agr*  
2 either directly or indirectly represses K-WTA in order to reduce total WTA surface  
3 density. Further, the shorter WTA chain length resulting when *agr* is not active (*i.e.* early  
4 exponential growth) may facilitate surface exposure of other bona fide bacterial surface  
5 protein-based adhesins, collectively known as MSCRAMMs (microbial surface  
6 components recognizing adhesive matrix molecules) (9). DELETE This raises the  
7 possibility that not only length, but also WTA density, may be part of the *agr* regulon.  
8 This idea needs to be further explored, as it is becoming clear that *S. aureus* has the  
9 capability to dynamically modulate its WTA. DELETE

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**Figure 1.** Genetic organization of WTA determinants in *S. aureus*. A) The WTA locus of *S. aureus* is predicted to contain at least three operon transcripts [*tarI'J'K*, *tarF*, and *tarIJJ(X)*] (43). The ~3.4 kb duplication regions are underlined, while the bracket marks the strain dependent intergenic region that separates *tarI'J'K* from the rest of the locus. B) Titration of TarL levels with IPTG inducer confirms *tarL* is an essential gene for growth of JT16 *in vitro* ( ). A stationary phase culture of JT16 grown in TSB supplemented with 1 mM IPTG was diluted (1:200) into fresh TSB containing various amounts of IPTG and incubated for 24 hrs at 30 °C before measuring culture turbidity. Blocking the first step of WTA biosynthesis by deleting *tarO* relieves growth dependence on TarL in the otherwise isogenic strain JT22( ).

**Figure 2.** Deletion of *tarI'J'K* genes does not influence phage binding but does influence WTA composition. A) The plaque forming efficiency of ø11 (clear bars), ø80 (solid bars), and ø85 (hatched bars) was measured using wildtype (RN4220), *tarI'J'*(JT17), and *tarK*(JT18) as recipient strains. PFU values were normalized to the number of plaques observed using wildtype RN4220 and the standard error represents data from three separate trials. B) PAGE analysis of isolated WTA extracts that have been visualized with alcian blue-silver staining (see Materials and Methods). Wildtype (RN4220), *tarO* (RN4220 *tarO*), *tarI'J'*(JT17), *tarK*(JT18), *P<sub>cad</sub>tarK* (JT19) with no inducer and 1 M Cd<sup>2+</sup>. Arrows denote regularly spaced intervening bands of K-WTA.

**Figure 3.** TarK and TarL promoter analysis and WTA gene complementation. A) The expression of *luxABCDE* from pXen-1 using the DNA promoter region fragments [JT204 *tarI'JK*( ), JT205 *tarIJJL*( ), or JT203 control ( )] was measured during early log growth. LCPS-relative luminescence counts per second. B) The  $\phi$ 11 mediated transduction efficiency of the *tarL::tetL* allele into recipient strains expressing the denoted WTA gene was determined by counting viable Tc<sup>r</sup> colonies after a 48 hour incubation.  $\phi$ 11-JT200, *S. aureus* TarL(JT208), *S. aureus* TarK(JT209), *B. subtilis* W23 TarK(JT210), *B. subtilis* W23 TarL(JT211), *B. subtilis* W23 TarKL(JT212). C) WTA was extracted from the complemented *tarL::tetL* transductants and analyzed by PAGE.  $\phi$ 11-JT200, *tarK*(JT207), TarL(JT213), TarK (JT214), TarKL<sup>W</sup>(JT215), Bs W23-*B. subtilis* W23 WTA.

**Figure 4.** Analysis of the *agr* phenotype. A) The secretion of hemolysins was qualitatively assessed on TSA blood agar plates. The inner halo of clearing is due to the *agr* RNAIII transcript which encodes  $\alpha$ -hemolysin (1). B) The extracellular protein fraction obtained from an equal number of cells was separated by SDS-PAGE and stained with Coomassie blue. C) Western blot of extracellular protein fractions probed with anti  $\alpha$ -hemolysin antibody. Approximately 5  $\mu$ g of protein was loaded for each sample.

1 **Figure 5.** The *agr* system affects WTA length and heterogeneity. PAGE profile of  
2 WTA extracts from wildtype strains and the corresponding *agr::tetM* null deletion strains.  
3 Repair of the *agr* defect in RN4220 represses K-WTA and increases average chain length  
4 of L-WTA. *agr tarK*(JT221), *agr*<sup>+</sup> (JT38).

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6  
7  
8 **Figure 6.** A) A revised model of polyRboP WTA biosynthesis in *S. aureus* that  
9 incorporates a bifunctional TarL RboP priming and polymerizing enzyme. The validated  
10 two enzyme model for *B. subtilis* W23 is shown for comparison. The branch point for  
11 the *agr* repressed K-WTA pathway is arbitrary as the structure is unknown (see text). The  
12 addition of a single GroP unit is shown for TarF to correlate with the observed activity of  
13 recombinant enzyme (6). Und-P- undecaprenyl phosphate. B) Chemical structure of the  
14 acceptor substrate during addition of CDP-Rbo to GroP in the WTA linkage unit, to  
15 RboP in the WTA chain, and to GroP in lipoteichoic acid (LTA). Note the differing  
16 stereochemistry of the secondary alcohol at the -carbon among the *in vivo* (GroP WTA,  
17 RboP WTA) and *in vitro* acceptors (boxed).

Table1: Bacterial strains

Strain	Relevant Genotype	Source or Reference
<i>S. aureus</i>		
RN450	NCTC 8325-4, prophage cured, <i>rsbU agr</i> <sup>+</sup>	(33)
RN4220	RN450 <i>r</i> <sup>-</sup> <i>m</i> <sup>+</sup> , partial <i>agr</i> defect	(27)
RN4220	RN4220 <i>tarO</i>	(19)
<i>tarO</i>		
RN6911	RN450 <i>agr::tetM</i> null Tc <sup>r</sup>	(35)
SA113	NCTC8325(Ø11,Ø12,Ø13) <i>r</i> <sup>-</sup> <i>m</i> <sup>+</sup> <i>agr</i> <sup>-</sup>	ATCC 35556
SH1000	RN450 <i>rsbU</i> <sup>+</sup> <i>agr</i> <sup>+</sup>	(22)
JT200	RN450(Ø11)	This study
JT15	RN4220 [ <i>geh::</i> (pCL25int-P <sub>spank</sub> <i>tarL</i> )Em <sup>r</sup> ] [SAOUHSC_00227]	This study
JT16	JT15 <i>tarL::tetL</i> Tc <sup>r</sup>	This study
JT17	RN4220 <i>tarI'J'</i> [SAOUHSC_00220-SAOUHSC_00221]	This study
JT18	RN4220 <i>tarK</i> [SAOUHSC_00222]	This study
JT19	JT18 (pMS74-P <sub>cas</sub> <i>tarK</i> ) Em <sup>r</sup>	This study
JT20	JT18 [ <i>geh::</i> (pCL25int-P <sub>pen</sub> <i>tarK</i> )Em <sup>r</sup> ]	This study
JT21	JT20 <i>tarL::tetL</i> Tc <sup>r</sup>	This study
JT22	RN4220 <i>tarO</i> [ <i>geh::</i> (pCL25int-P <sub>spank</sub> <i>tarL</i> )Em <sup>r</sup> ] <i>tarL::tetL</i> Tc <sup>r</sup> by transduction (JT15 and then JT16 as donor)	This study
JT38	RN4220 (pCN33- <i>agrA</i> ) Em <sup>r</sup>	This study
JT203	RN450 (pXen-1) Cm <sup>r</sup>	This study
JT204	RN450 (pXen-P <sub><i>tarI'JK</i></sub> ) Cm <sup>r</sup>	This study
JT205	RN450 (pXen-P <sub><i>tarJLL</i></sub> ) Cm <sup>r</sup>	This study
JT207	JT200 <i>tarK</i> [SAOUHSC_00222]	This study
JT208	JT207 [ <i>geh::</i> (pCL25int-P <sub>pen</sub> <i>tarL</i> )Em <sup>r</sup> ]	This study
JT209	JT207 [ <i>geh::</i> (pCL25int-P <sub>pen</sub> <i>tarK</i> )Em <sup>r</sup> ]	This study
JT210	JT207 [ <i>geh::</i> (pCL25int-P <sub>pen</sub> <i>tarK</i> <sup>Bs</sup> )Em <sup>r</sup> ]	This study
JT211	JT207 [ <i>geh::</i> (pCL25int-P <sub>pen</sub> <i>tarL</i> <sup>Bs</sup> )Em <sup>r</sup> ]	This study
JT212	JT207 [ <i>geh::</i> (pCL25int-P <sub>pen</sub> <i>tarKL</i> <sup>Bs</sup> )Em <sup>r</sup> ]	This study
JT213	JT208 <i>tarL::tetL</i> Tc <sup>r</sup> by transduction (JT16 donor)	This study
JT214	JT209 <i>tarL::tetL</i> Tc <sup>r</sup> by transduction (JT16 donor)	This study
JT215	JT212 <i>tarL::tetL</i> Tc <sup>r</sup> by transduction (JT16 donor)	This study
JT220	JT200 <i>agr::tetM</i> null Tc <sup>r</sup> by transduction (RN6911 donor)	This study
JT221	JT207 <i>agr::tetM</i> null Tc <sup>r</sup> by transduction (RN6911 donor)	This study
JT302	SH1000 <i>agr::tetM</i> null Tc <sup>r</sup> by transduction (RN6911 donor)	This study
JT409	SA113 <i>agr::tetM</i> null Tc <sup>r</sup> by transduction (RN6911 donor)	This study
<i>B. subtilis</i>		
W23	Prototype PolyRboP WTA strain	ATCC 23059

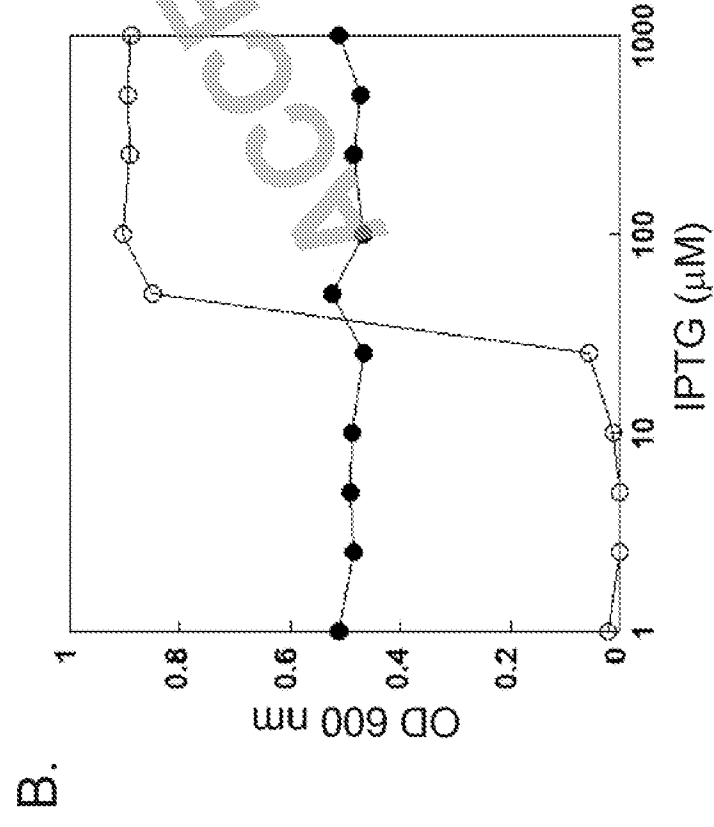
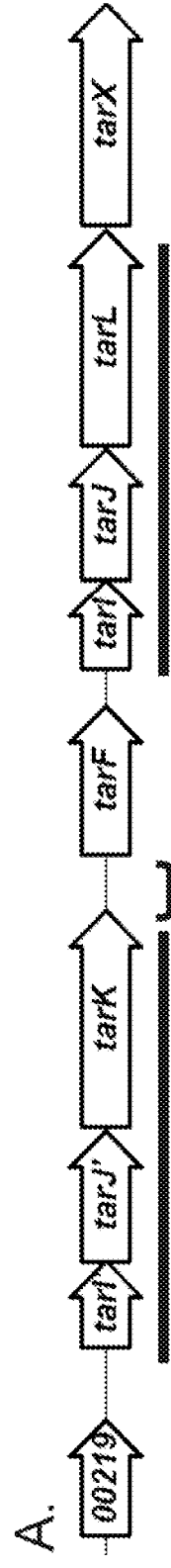
Table 2: Plasmids and primers

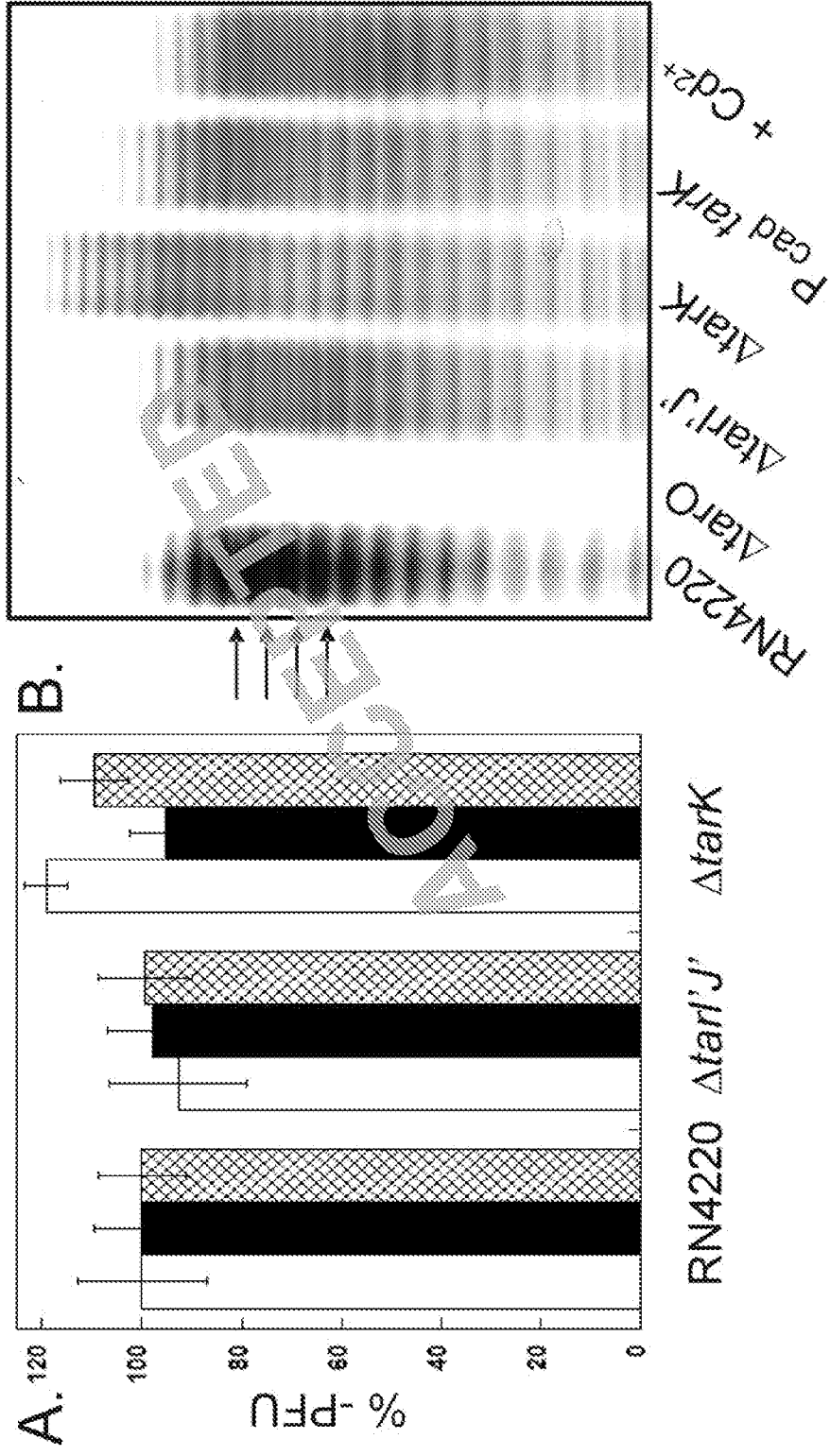
Plasmid/ primer	Description <sup>a</sup>	Source or Reference
pKOR1	<i>E. coli</i> / <i>S. aureus</i> shuttle vector <i>ori</i> <sup>TS</sup> inducible <i>secY</i> -antisense counterselection Ap <sup>r</sup> Cm <sup>r</sup>	(4)
pCN33	<i>E. coli</i> / <i>S. aureus</i> shuttle vector Ap <sup>r</sup> Em <sup>r</sup>	(7)
pCN59	<i>E. coli</i> / <i>S. aureus</i> shuttle vector P <sub>cad</sub> <i>cadC blaZ</i> TT Ap <sup>r</sup> Em <sup>r</sup>	(7)
pCN68	<i>E. coli</i> / <i>S. aureus</i> shuttle vector P <sub>blaZ</sub> GFP <i>blaZ</i> TT Ap <sup>r</sup> Em <sup>r</sup>	(7)
pXen-1	<i>E. coli</i> / <i>S. aureus</i> shuttle vector <i>luxABCDE</i> Cm <sup>r</sup>	Xenogen
pCL25	Phage L54a <i>attP-attB</i> ( <i>geh</i> ) integration vector Spec <sup>r</sup> Te <sup>r</sup>	(30)
pMS13	pKOR1 <i>tarO::tetL</i> allelic replacement vector	This study
pMS17	pKOR1 <i>tarI'J'</i> allelic replacement vector	This study
pMS18	pKOR1 <i>tarK</i> allelic replacement vector	This study
pMS21	pKOR1 <i>tarL::tetL</i> allelic replacement vector	This study
pCL25int	pCL25 with phage 54a integrase and <i>ermC</i> cassette replacing <i>tetL</i> Spec <sup>r</sup> Em <sup>r</sup>	This study
pCL25intP <sub>spank</sub>	pCL25int with P <sub>spank</sub> IPTG inducible promoter P <sub>pen</sub> <i>lacI</i>	This study
pCL25intP <sub>Pen</sub>	pCL25int with P <sub>Pen</sub> constitutive promoter <i>blaZ</i> TT	This study
pMS74	pCN59 with low copy p1258 replicon Ap <sup>r</sup> Em <sup>r</sup>	This study
pMS80	<i>E. coli</i> / <i>S. aureus</i> shuttle vector pLI50 with <i>tarO</i> Ap <sup>r</sup> Cm <sup>r</sup>	This study
Primers		
P1tarK	Gattacatgggcccgatctagtggttatg	
P2tarK	Ctggatcatgataaacaataaatattatc	
P3tarK	Gttatcatgaccaggaaaaagtgtgcca	
P4tarK	Gattacatcccggcgatgtcgaatatgg	
P1tarI'J'	Gattacatgggcccgaacatcattgggcg	
P2tarI'J'	Aatctcattaaattcactcactaaaatgaac	
P3tarI'J'	Gaatttaatgagattaatgtatgtacgatg	
P4tarI'J'	Tggtgcctttaaaacagtgg	
P1tarL	Gattacatgggcccctagacaatttgaag	
P4tarL	Catctacaatatcaaaacggtagtattgacc	
tetL-for	Gcttagttcgaatggggaaagcttcacaga	
tetL-rev	Gcttgaaacgcgttaagtctaacacactagac	
tarLspank-for	Ggctatgtcgaccagtattaaaatggattatg	
tarLspank-rev	Gctacagctagcactttgactactatataaac	
agrA-F	Tgttatgcatgcctggcctacgtgattatttc	
agrA-R	Tgttcgaattcacgcgtcataatttaattttg	
TarO-F	Gctatctgggcccgaataataaatgc	
TarO-R	Gctatctcgtacgcctaaaatatactcatagc	
ProtarI'J'K-for	Gcttatgaattcgtatgcacctttgaggacg	
ProtarI'J'K-rev	Gcttatggatccgttgctctccattctgtc	
ProtarIJL-for	Gcttatgaattcgtgataacttaaaaac	
ProtarIJL-rev	Gcttatggatccaaaatacttctccattc	

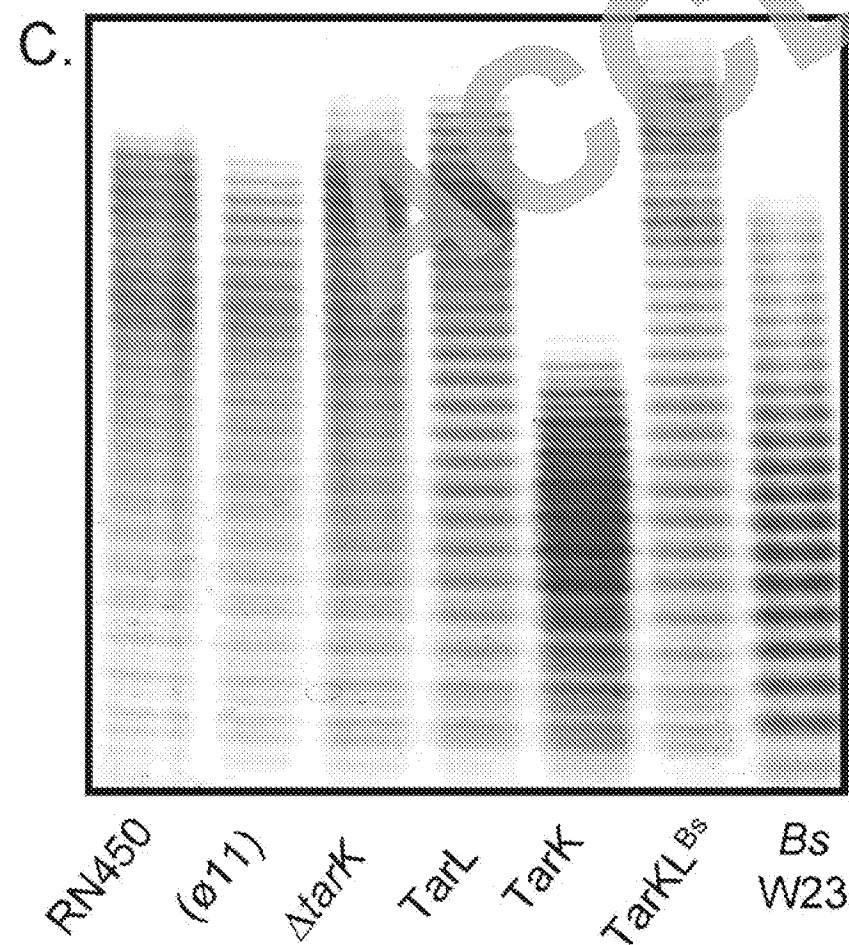
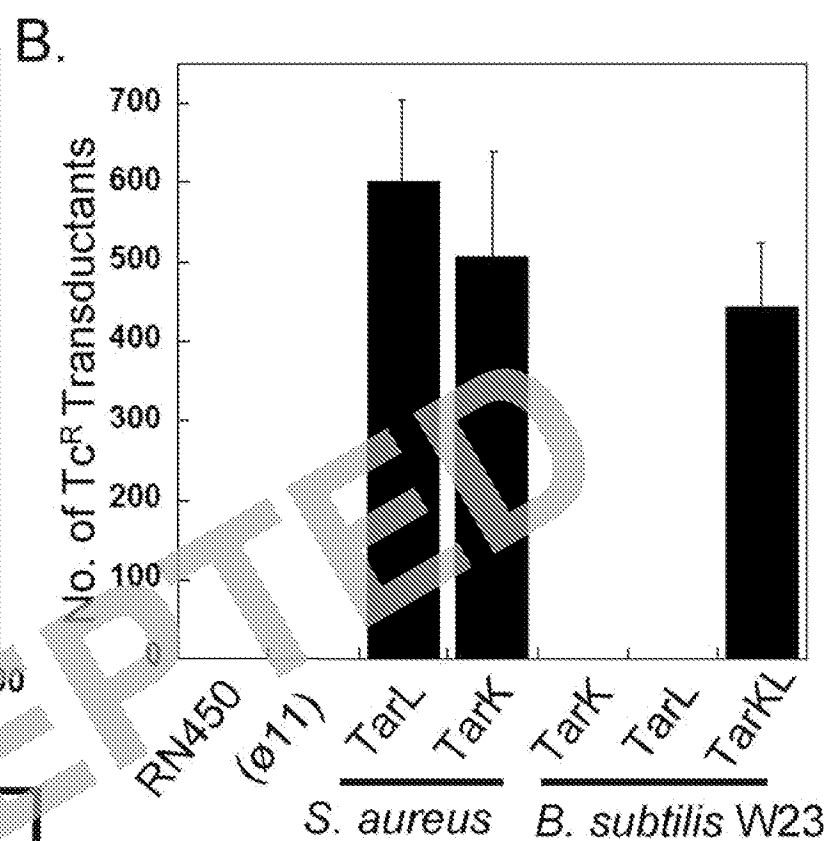
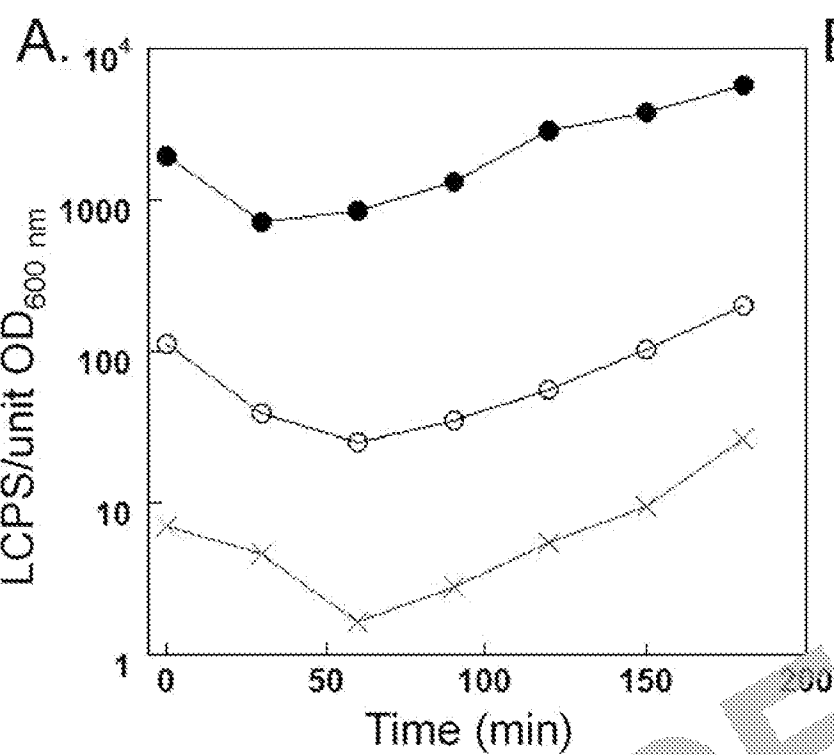


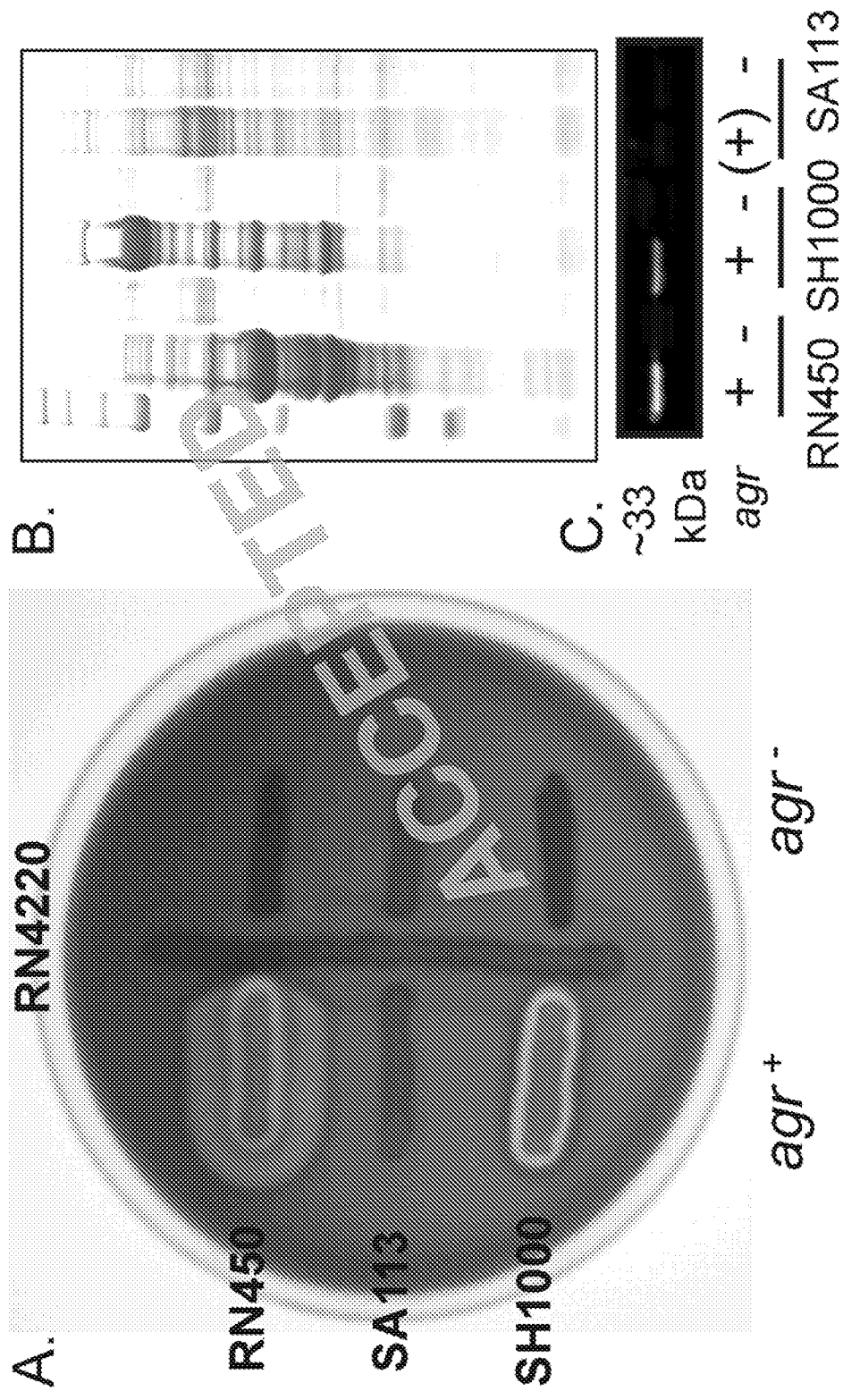
Ppenfor	Gacctgcaggcatgcaagetaattcc
Ppenrev	gacggtcgtcgacaatatttgattgatcgtaaccag
tarLpen-for	cgtagaattc <u>gaggaggag</u> taaaagtATGGTTAAAAGTAAGAT
tarLpen-rev	gggccgcggcgccTTAGCTACCAAATAAATTTC
tarKpen-for	cgtagaattc <u>gaggaggag</u> taaaagtATGACAAAAACGAAACAAGC
tarKpen-rev	gaccgtcgagctcccaacaTTAGCGTCTAAACAAATC
tarLBs pen-for	cctatggtcgcac <u>gaggaggag</u> taaaagtATGAAGCTGGCCAG
tarLBs pen-rev	gggccgcggcgccTTATCTTTTAAGGACTTTATC
tarKBs pen-for	cctatggtcgcac <u>gaggaggag</u> taaaagtATGAAAACATTCCTTAC
tarKBs pen-rev	gggccgcggcgccCTAATCGGCACCAGATG

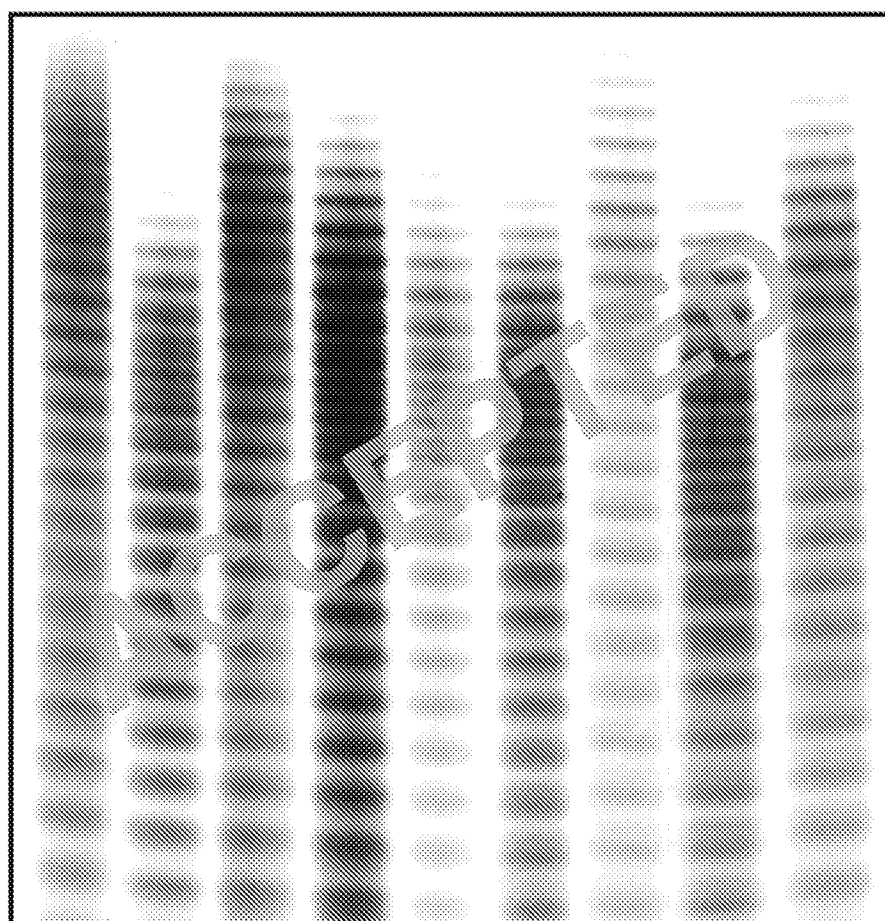
<sup>a</sup> Restriction sites are italicized, synthetic ribosome binding sites underlined, and gene coding sequences in upper case.











<i>agr</i>	+	-	+	-	(+)	-			
	<u>          </u>		<u>          </u>		<u>          </u>				
	RN450		SH1000		SA113		$\Delta agr$ <i>tark</i>	RN4220	<i>agr</i> <sup>+</sup>

